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14. ABSTRACT
Beclin 1, the mammalian homologue of the yeast Atg6, is a key autophagy - promoting gene that plays a critical role in the regulaticell death and survival of various types of cells. However, recent studies have observed that the expression of beclin 1 is altercertain diseases including cancers. The causes underlying the aberrant expression of beclin 1 remain largely unknown. We report that micro RNAs (miRNAs), a class of endogenous, 22 - 24 nucleotides non-coding RNA molecules able to affect stability translation of mRNA, may represent a previously unrecognized mechanism for regulating beclin 1 expression and autophagy. demonstrated that beclin 1 is a potential target for miRNA miR-30a, and this miRNA could negatively regulate beclin 1 expre resulting in decreased autophagic activity. Treatment of tumor cells with the miR-30a mimic decreased, and with the antag increased, the expression of beclin 1 mRNA and protein. Dual luciferase reporter assay confirmed that the miR-30a binding seque in the 3' - UTR of beclin 1 contribute to the modulation of beclin 1 expression by miR-30a. Furthermore, inhibition of bec expression by the miR-30a mimic blunted activation of autophagy induced by rapamycin. Our study of the role of miR-30regulating beclin 1 expression and autophagy reveals a novel function for miRNA in a critical cellular event with significant impac

15. SUBJECT TERMS
beclin 1; autophagy; microRNA; miR-30a; gene expression

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INTRODUCTION

Autophagy, a conserved, programmed response to metabolic and environmental stress found in yeast, plants, worms, flies, mice, and man [1], has been known to play a critical role in the regulation of survival and death of various types of cells [2]. Recent studies have implicated autophagy in a number of physiologic and pathophysiologic processes such as aging, cancers, and neurodegenerative diseases [3]. The process of autophagy involves formation of double-membrane vesicles (autophagosomes) that engulf organelles and cytoplasm, then fuses with the lysosome to form the autolysosme, where the contents are degraded and recycled for protein and ATP synthesis [4, 5]. The formation of the autophagosome is mediated by a series of autophagy - promoting gene products that function at different stages of autophagy [6]. *Beclin 1*, the mammalian homologue of the yeast *Atg6*, is a key autophagy - promoting gene whose product is part of a lipid kinase (class III Phosphoinositide 3-kinase) complex that participates in the early stage of autophagosome formation [7, 8]. Beclin 1 is a ~60-kDa coiled – coli protein also able to interact with bcl-2, an anti-apoptotic protein. Although ubiquitously expressed, it has been known that the expression of beclin 1 is altered in certain diseases. For example, in early Alzheimer disease beclin 1 expression is decreased [9]; in contrast, neurodegeneration causes up-regulation of beclin 1 [10]. In several types of human cancers, the expressions of *beclin 1*, both protein and mRNA, were also found to be aberrant [11-13]. Yet, the causes underlying the altered expression of this key autophagy – promoting gene remains largely unknown. In the present study we sought to explore the role of microRNAs (miRNAs) in the regulation of expression of *beclin 1*. MiRNAs are a class of endogenous, 22 - 24 nucleotides RNA molecules with the ability to induce mRNA degradation, translational repression, or both, via pairing with partially complementary sites in the 3' UTR of the targeted genes [14-17]. It is estimated that over 1000 miRNAs exist in mammalian cells, and that 30% of all genes are regulated by miRNAs [18-20]. Because of their capacity to target numerous mRNAs, miRNAs can regulate the expression of genes in a number of pathways that are associated with tumor initiation, development and progression [21-23]. During this grant period, we discovered that the autophagy - promoting gene *beclin 1* is a potential target for miR-30a, and this miRNA may play a regulatory role in autophagic response through modulating the expression of *beclin 1*.

BODY

Task 1 To determine the importance of autophagy in the life and death of breast cancer cells.

Task 2 To determine the role of autophagy in the sensitivity of breast cancer to treatment.

During the last grant period, we focused on the roles of miRNA in regulating autophagy. We first analyzed the miRNA expression profiling in tumor cells treated with HBSS (nutrient deprivation) or rapamycin. Nutrient deprivation and rapamycin treatment are known to

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activate autophagy in various types of cells [24, 25]. Using a miRNA microarray (OSUCCC-microRNA version 4.0) analysis, we observed a differential expression of 13 miRNAs in T98G and MDA-MB-468 cells treated with HBSS or rapamycin, as compared with the untreated cells or the cells treated with vehicle. The heat map of these miRNA expression profiles of those samples is shown as supplemental data (Figure 1). We then conducted an *in silico* search for miRNA binding sites using the PicTar algorithm (http://pictar.bio.nyu.edu). Among those 13 miRNAs that were differentially expressed in the HBSS or rapamycin – treated cells, we found in the 3' - UTR of *beclin 1* the consensus sequences for miR-30a, implying that *beclin 1* is a potential target for miR-30a. To verify the change of miR-30a expression following HBSS or rapamycin treatment, we performed qRT-PCR analysis of the endogenous miR-30a expression. Fig. 2 shows that there was a 10% and 35% reduction of miR-30a expression in cells subjected to nutrient depletion or rapamycin treatment, respectively. These results suggest a possible role for this miRNA in targeting *beclin 1* in response to stresses.

To obtain experimental evidence supporting *beclin 1* gene as a target for miR-30a, we next examined the effect of miR-30a on *beclin 1* expression using a mimic and an antagomir of this miRNA. In these experiments, T98G, MDA-MB-468 and H1299 cells were transfected with a miR-30a mimic or an antagomir, and then the expressions of *beclin 1* mRNA and protein were analyzed by qTR-PCR and Western blot, respectively. As shown in Fig. 3, transfection with the miR-30a mimic caused a 25-40% decrease in *beclin 1* mRNA (Fig. 3A), and a 25-60% decrease in beclin 1 protein (Fig. 2B); by contrast, treatment with the miR-30a antagomir resulted in a 55-85% increase in *beclin 1* mRNA (Fig. 2A) and a 10-25% increase in beclin 1 protein (Fig. 3B). Using the mimic and antagomir of miR-30a, these experiments demonstrated a suppressive role for this miRNA in *beclin 1* expression.

To validate the predicted consensus sequences for miR-30a in the *beclin 1* – 3' UTR, and determine whether these miR-30a binding sequences directly contributed to the negative regulation of *beclin 1* expression, we tested the effects of miR-30a on activity of a reporter gene using the vectors that either contained wild-type miR-30a targeting sequences (psiCHECKTM2-WT-*BECN*-3'-UTR) or deletion mutant (psiCHECKTM2-MT-*BECN*-3'-UTR) (Fig. 4B). As shown in Fig. 4C, co-transfection of T98G, MDA-MB-468 and H1299 cells with 50 nM or 100 nM of the miR-30a mimic resulted in a 40-80% reduction in the activity of the reporter gene vector containing the wild-type miR-30a targeting sequences (psiCHECKTM2-WT-*BECN*-3'-UTR), in comparison to that of the vector with the deletion mutant (psiCHECKTM2-MT-*BECN*-3'-UTR). In contrast, the non-targeting control RNA did not have any effect on the reporter activity of either of the vectors (Fig. 4C). These results demonstrated that the miR-30a binding sequences in the *beclin 1* – 3' UTR is the region required for the miR-30a - mediated inhibition of *beclin 1* expression.

Beclin 1 plays an essential role in activating autophagy. To determine the functional consequence of modulation of *beclin 1* expression by miR-30a, we tested the effect of the miR-30a mimic on autophagic response to rapamycin. Fig. 5 shows that treatment of T98G cells with rapamycin activated autophagy, as evidenced by the increases in LC3-II amount (Fig. 5A), in double membrane vacuoles in the cytoplasm (Fig. 5B), and in formation of GFP-LC3 aggregation (Fig. 5C). Notably, tumor cells transfected with the mimic of miR-30a showed a remarkable reduction in beclin 1 expression, and a blunted autophagic response to rapamycin,

as evidenced by the lower LC3-II levels (Fig. 5A), fewer double membrane vacuoles (Fig. 5B), and less GFP-LC3 aggregations (Fig. 5C), indicating that inhibition of *beclin 1* expression by miR-30a leads to suppression of autophagic activity. To confirm that the effect of miR-30a on autophagy was mediated through beclin 1, we tested the effect of miR-30a on the resveratrol – induced autophagy in MCF-7 cells, which was reported to be beclin 1-independent [26]. Fig. 6 shows that miR-30a mimic had no effect on autophagy induced by resveratrol in MCF-7 cells.

KEY RESEARCH ACCOMPLISHMENTS

•   We found that *beclin 1* is a putative target for miR-30a.

•   We showed that miR-30a negatively regulates *beclin 1* expression.

•   We demonstrated the effect and mechanism of miR-30a on autophagic activity.

REPORTABLE OUTCOMES

Manuscript
> Zhu H, Wu H, Liu XP, Li B, Chen Y, Ren XC, Liu CG, Yang JM: Regulation of Autophagy by a *Beclin 1*-targeted MicroRNA, miR-30a, in Cancer Cells. Autophagy 2009 Aug 20; 5(6). [Epub ahead of print]

Abstracts
> Zhu H, Wu H, Liu X, Chen Y, Ren XC, Liu CG, Yang JM: Regulation of Autophagy by the *Beclin 1*-targeted MicroRNA in Cancer Cells.   Proc Amer Assoc Cancer Res 50: 387, 2009.

Degree obtained that are supported by this award
None

CONCLUSIONS

Our study of the role of miR-30a in regulating *beclin 1* expression and autophagy reveals a novel function for miRNA in a critical cellular event with significant impacts in cancer development, progression, and treatment.

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APPENDIX

Figure 1. Heat map of miRNAs expression profiles of the samples subjected to nutrient deprivation.
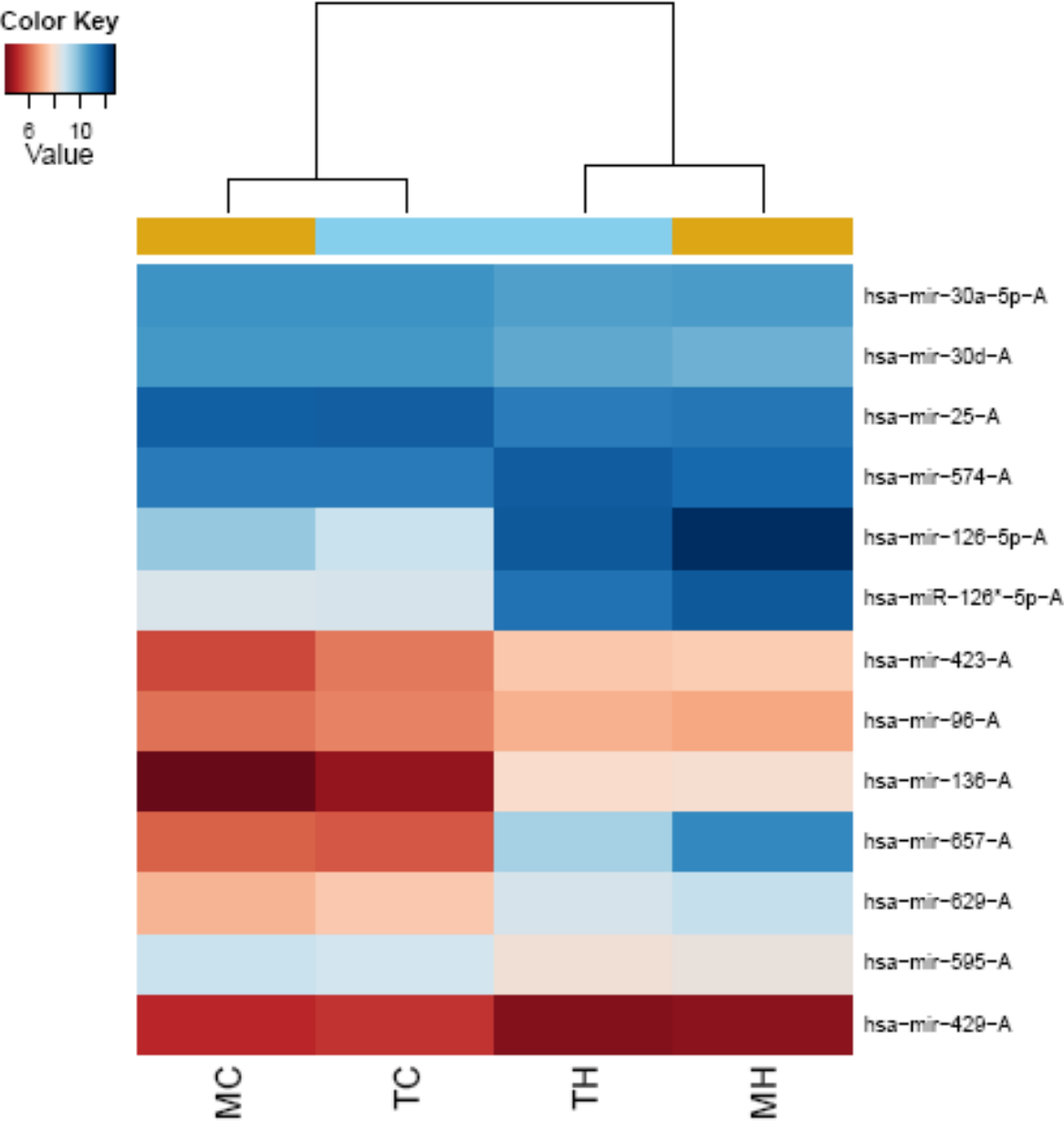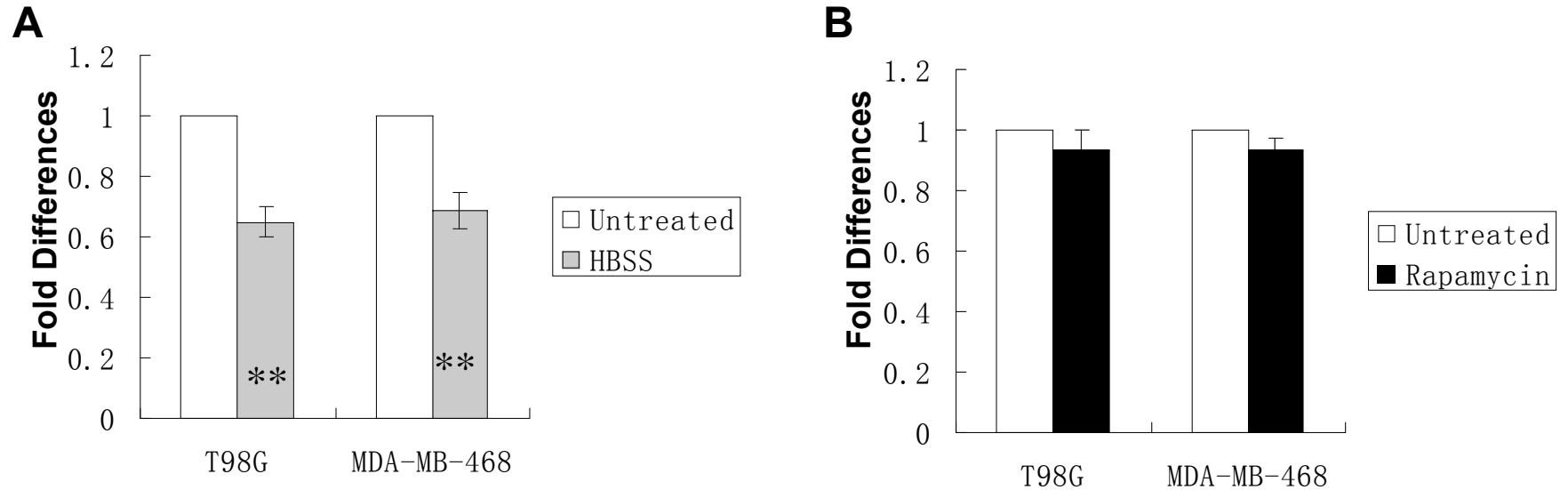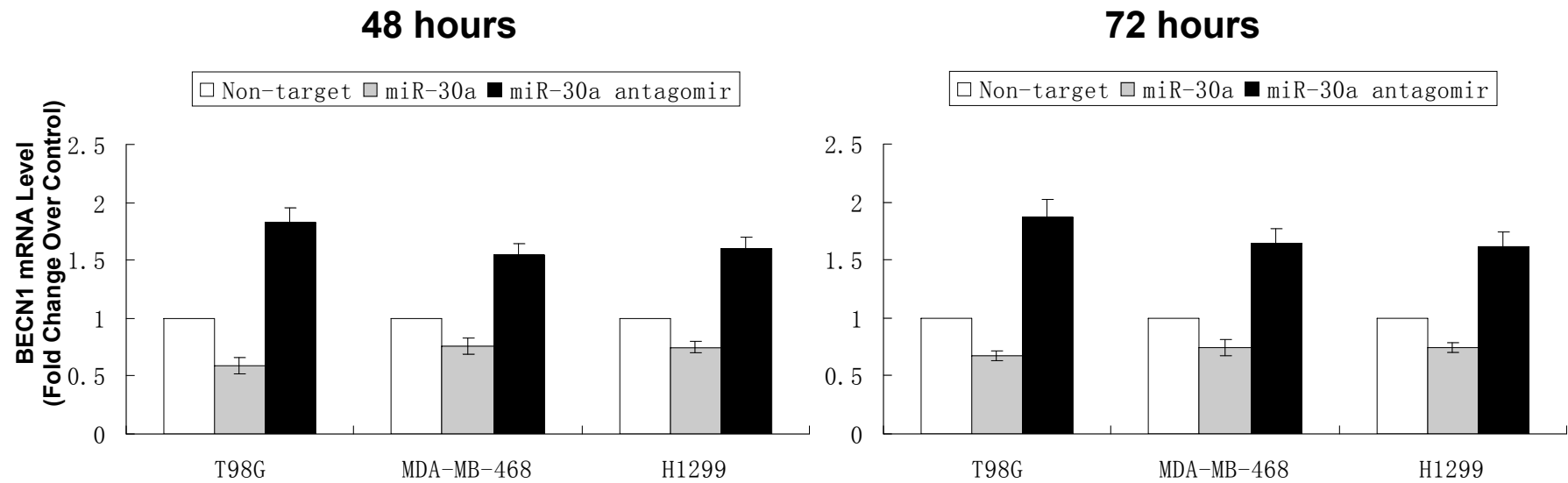
Figure 2

Figure 2. Effects of HBSS and rapamycin on endogenous miR-30a expression. T98G and MDA-MB-468 cells were treated with HBSS for 4 h or rapamycin (200 nM) for 12 h. At the end of treatment, endogenous miR-30a expression was analyzed by Real-time RT-PCR. Small nuclear RNA (RNU66) was used as internal control. The expression level of miR-30a was calculated using the MxPro software (Version 4.00, Stratagene). Results shown are the mean ⅂ SD of triplicate determinations from one of three identical experiments. * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control

A

48 hours

72 hours

Figure 3

B

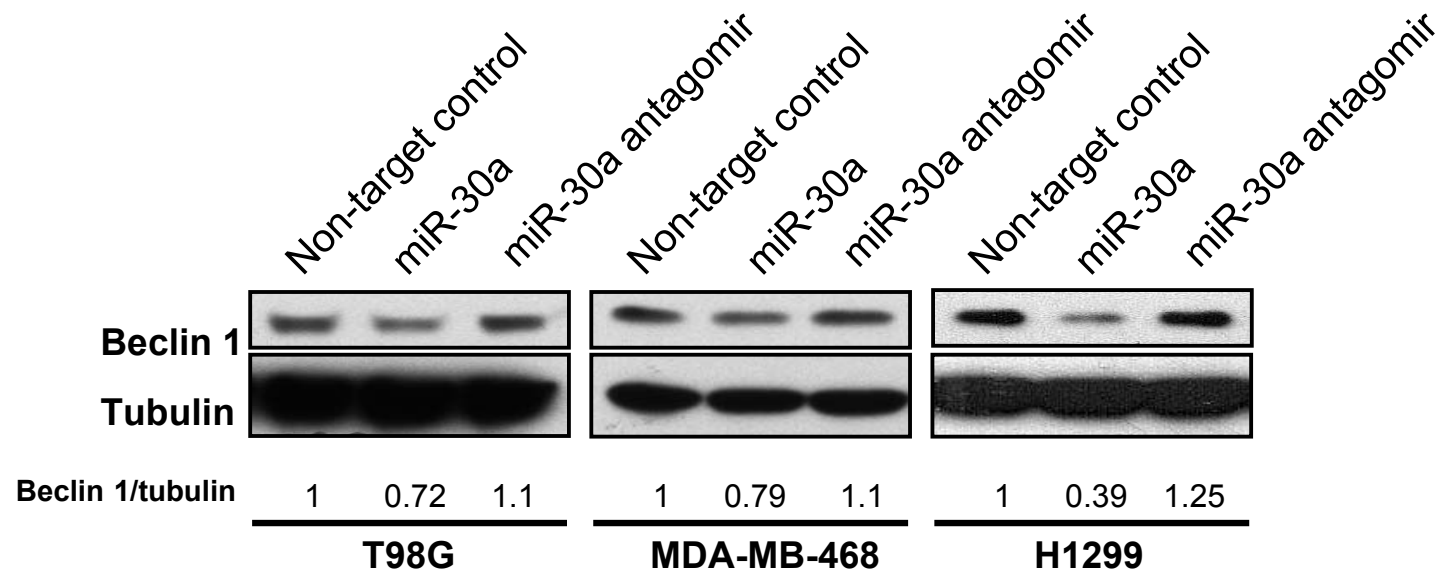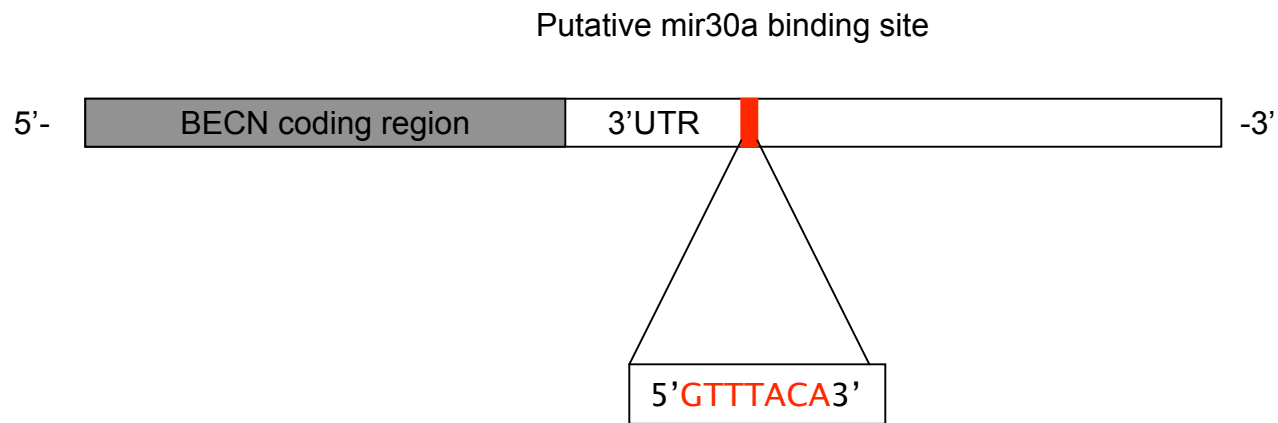Beclin 1/tubulin   1   0.72   1.1   1   0.79   1.1   1   0.39   1.25

Figure 3. Effect of miR-30a on the expression of *beclin 1*. **(A)** T98G, MDA-MB-468 and H1299 cells were transfected with a mimic or antagomir of miR-30a (100 nM) or a control RNA (100 nM). Forty-eight and 72 hours later, total RNAs were extracted from the treated cells and quantitative real-time RT-PCR analyses of *beclin 1* mRNA were performed. *Beclin 1* mRNA levels of the cells treated with a control RNA were arbitrarily set at 1, and *beclin 1* mRNA levels of the cells treated with the miR-30a mimic were normalized to the control. Results shown are the mean ∓ SD of triplicate determinations from one of three identical experiments. **(B)** T98G, MDA-MB-468, and H1299 cells were treated as described in (A). Forty - eight hours later, cell lysates were prepared from the transfected cells. Equal amounts (25 ∓g proteins) of cell lysates were separated by 8% SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were immunoblotted with a monoclonal anti-beclin 1 antibody. Detection of beclin 1 was performed using enzyme-linked chemiluminescence. α - tubulin was used as a loading control. Protein expression was quantified using the ImageJ software. Beclin 1/tubulin ratios of the samples treated with a control RNA was arbitrarily set at 1, and the beclin 1/ tubulin ratios of the miR-30a mimic or antagomir - treated samples were normalized to the control. Results shown are the mean ∓ SD of triplicate determinations from one of three identical experiments.
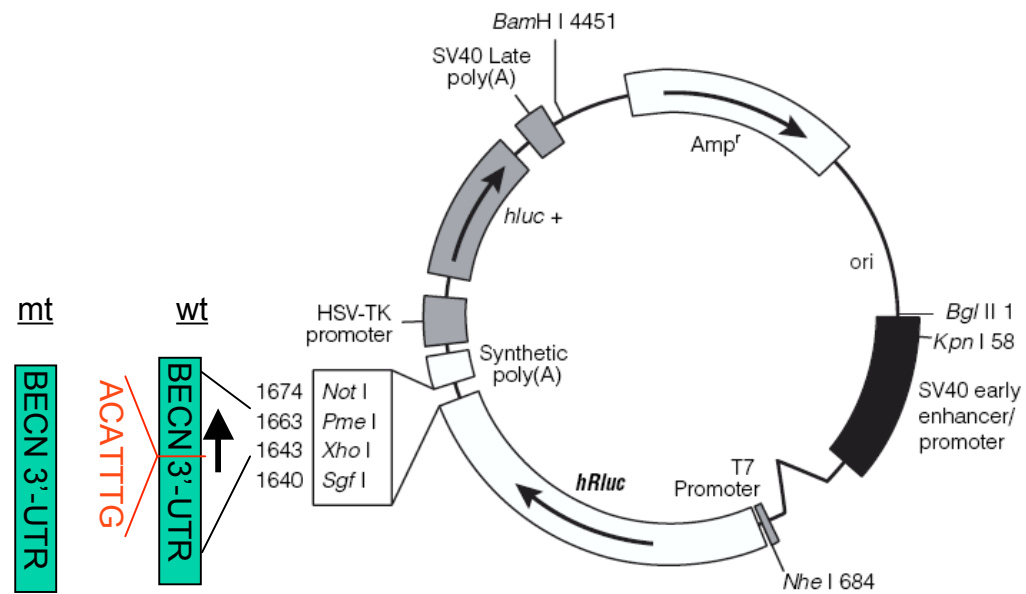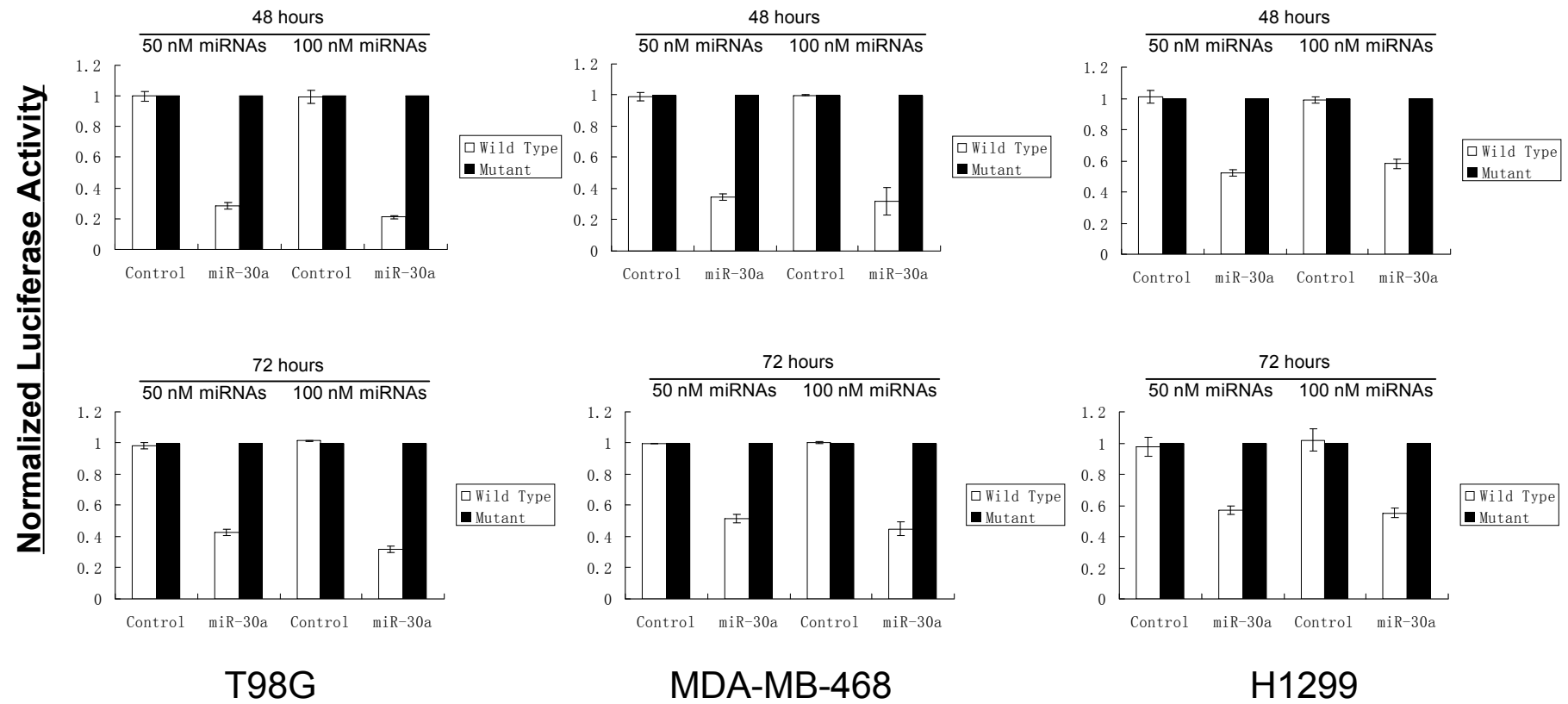
A

Figure 4

Putative mir30a binding site

5'- | BECN coding region | 3'UTR | | -3'

5'GTTTACA3'

B

BamH I 4451
SV40 Late poly(A)
Amp^r
hluc +
ori
HSV-TK promoter
Bgl II 1
Kpn I 58
SV40 early enhancer/ promoter
Synthetic poly(A)
1674 | Not I
1663 | Pme I
1643 | Xho I
1640 | Sgf I
hRluc
T7 Promoter
Nhe I 684

mt   wt

BECN 3'-UTR   ACATTTG   BECN 3'-UTR

Figure 4

Figure 4. *Beclin 1* **is a target for miR-30a.** **(A)** The miR-30a consensus sequences in the *beclin 1* 3' - UTR. **(B)** Construction of the psiCHECKTM2-WT-*BECN*-3'-UTR and psiCHECKTM2-MT-*BECN*-3'-UTR. The 1574 bp fragment of the *beclin 1* 3'-UTR containing the miR-30a consensus sequences was inserted into the psiCHECKTM2 dual luciferase reporter plasmid at the 3' end of the coding sequence of *R. reniformis* luciferase. The deletion mutant (psiCHECKTM2-MT-*BECN*-3'-UTR) was generated using mutagenesis PCR method. **(C)** Luciferase reporter assays. Cells were co-transfected with either psiCHECKTM2-WT-*BECN*-3'-UTR or psiCHECKTM2-MT-*BECN*-3'-UTR vector and a miR-30a mimic or a non-targeting control RNA. Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega), and *Renilla* luciferase activity was normalized to firefly luciferase activity. Results shown are the mean ⁷ SD of triplicate determinations from one of three identical experiments.
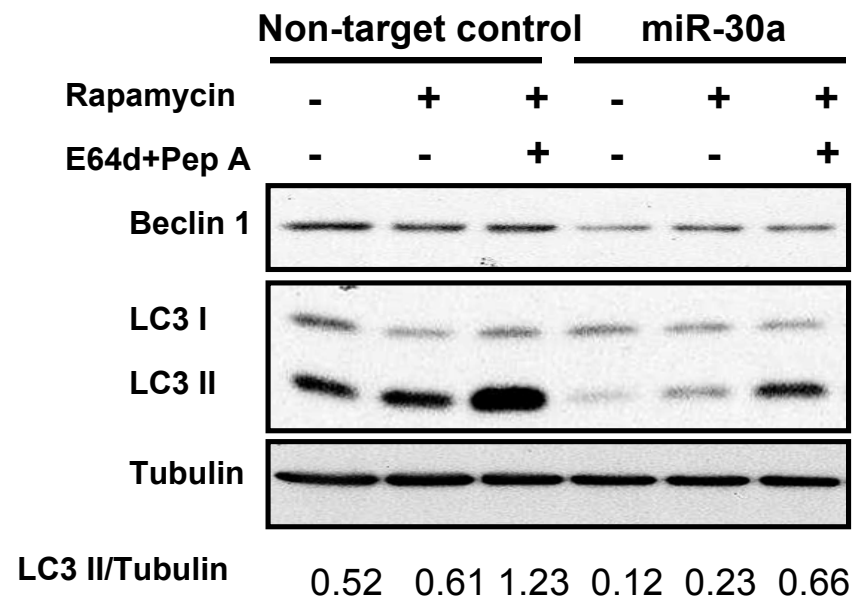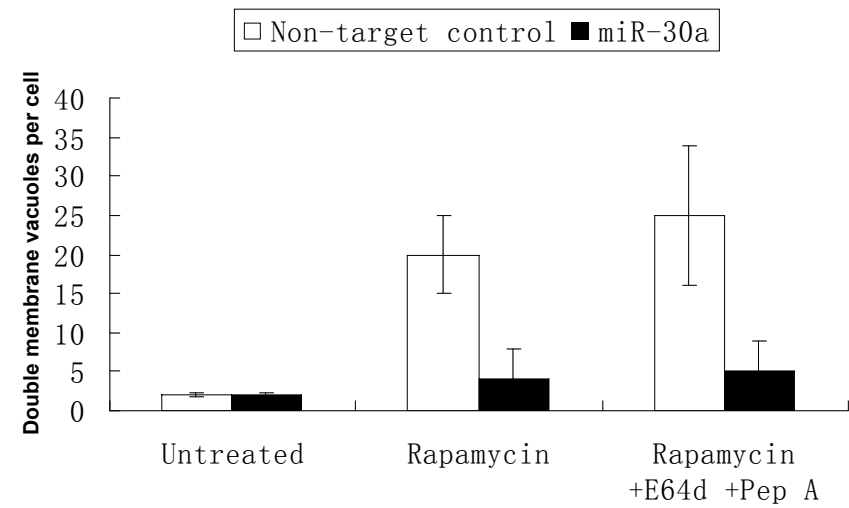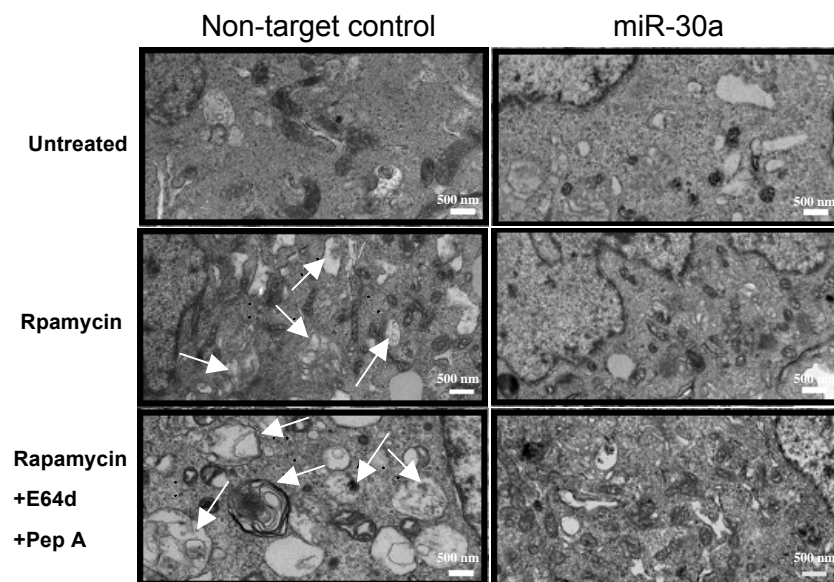
Figure 5

A

	Non-target control			miR-30a		
Rapamycin	-	+	+	-	+	+
E64d+Pep A	-	-	+	-	-	+
LC3 II/Tubulin	0.52	0.61	1.23	0.12	0.23	0.66

Beclin 1

LC3 I
LC3 II

Tubulin

Figure 5

B

Figure 5

C

Untreated | Rapamycin | Rapamycin+E64d+Pep A

Non-target control

miR-30a

10 μm

□ Non-target control ■ miR-30a

LC3-GFP puncta per cell

60
50
40
30
20
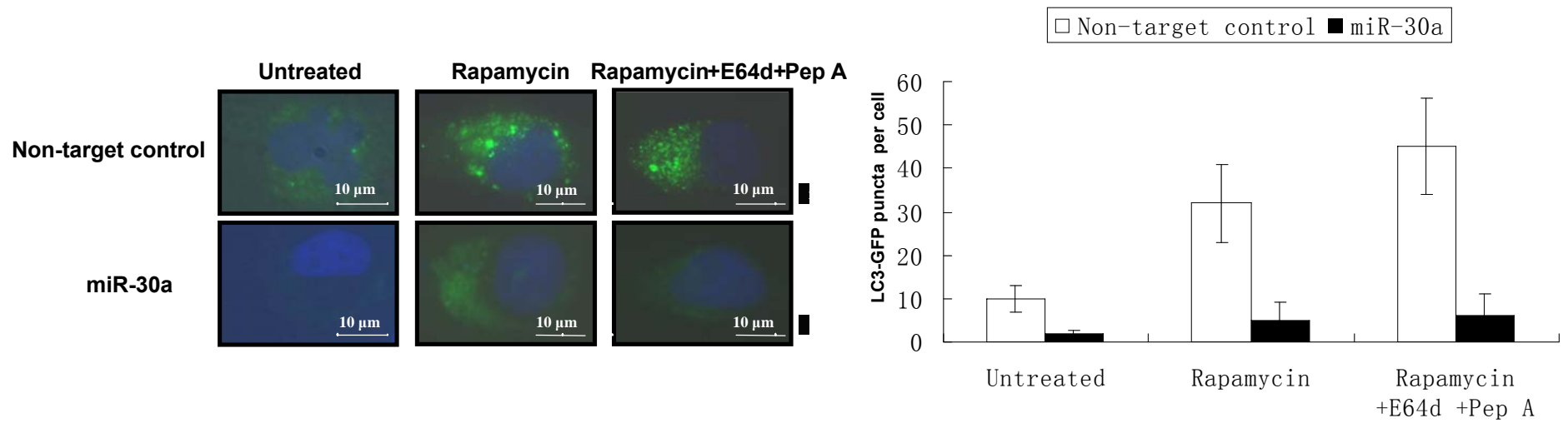10
0

Untreated | Rapamycin | Rapamycin +E64d +Pep A

Figure 5. Effect of miR-30a on autophagic response to rapamycin. **(A)** T98G cells transfected with a mimic of miR-30a or a non-targeting control RNA were treated with rapamycin (200 nM) in the presence or absence of lysosomal protease inhibitors E64D (10 ⱦg/ml) and pepstatin A (10 ⱦ g/ml). At the end of treatment, formation of LC3-II was detected by immunoblotting with an anti-MAP-LC3 antibody. **(B)** T98G cells were treated as described in (A), and then were harvested by trypsinization, fixed and embedded in spur resin. Ninety nm thin sections were cut and examined at 80 Kv with a JEOL 1200EX transmission electron microscope. Arrows indicate autophagic vacuoles. Double membrane vacuoles per cell were determined by counting 20 cells for each sample and average numbers of double membrane vacuoles were shown. The bars are the mean ⱦ S.D.. **(C)** GFP-LC3-expressing T98G cells were treated as described in (A). At the end of treatment, cells were fixed with 4% formaldehyde for 15min and inspected at 60x magnification for numbers of GFP-LC3 puncta. LC3-GFP puncta per cell were determined by counting 20 cells for each sample and average numbers of puncta per cell were shown. The bars are the mean ⱦ S.D.. Results shown are the representative of three identical experiments.
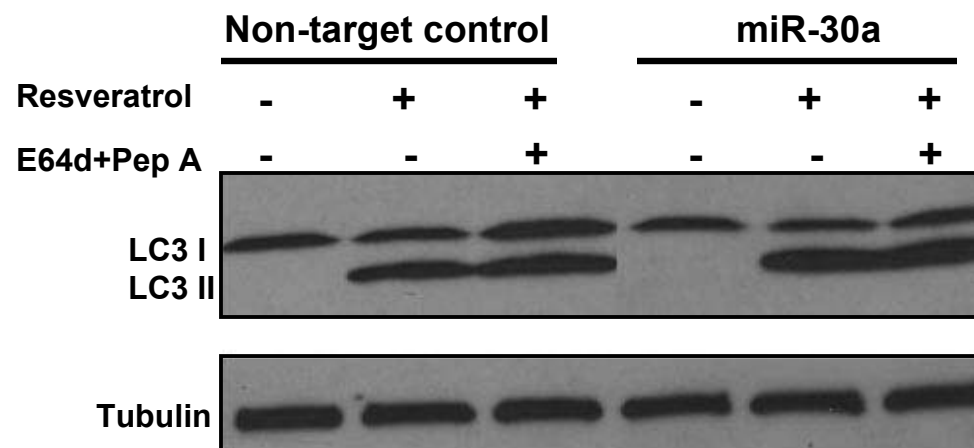
Figure 6

Figure 5. miR-30a does not affect the resveratrol-induced beclin 1-independent autophagy.
MCF-7 cells transfected with a mimic of miR-30a or a non-targeting control RNA were treated with resveratrol (50 ⱮM) in the presence or absence of lysosomal protease inhibitors E64D (10 Ɫg/ml) and pepstatin A (10 Ɫg/ml). At the end of treatment, formation of LC3-II was detected by immunoblotting with an anti- MAP-LC3 antibody.